ent characteristic (e.g., size, shape or color) that allows it to be detected without having to be conjugated to a separate label.

[0021] A "polymorphic marker" or "polymorphic site" is the locus at which divergence occurs. Preferred markers have at least two alleles, each occurring at frequency of greater than 1%, and more preferably greater than 10% or 20% of a selected population. A polymorphic locus may be as small as one base pair. Polymorphic markers include restriction fragment length polymorphisms, variable number of tandem repeats (VNTR's), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, and insertion elements such as Alu. The first identified allelic form is arbitrarily designated as the reference form and other allelic forms are designated as alternative or variant alleles. The allelic form occurring most frequently in a selected population is sometimes referred to as the wildtype form. Diploid organisms may be homozygous or heterozygous for allelic forms. A diallelic polymorphism has two forms. A triallelic polymorphism has three forms.

[0022] A "single nucleotide polymorphism" (SNP) occurs at a polymorphic site occupied by a single nucleotide, which is the site of variation between allelic sequences. The site is usually preceded by and followed by highly conserved sequences of the allele (e.g., sequences that vary in less than $1/100$ or $1/1000$ members of the populations). A single nucleotide polymorphism usually arises due to substitution of one nucleotide for another at the polymorphic site. A transition is the replacement of one purine by another purine or one pyrimidine by another pyrimidine. A transversion is the replacement of a purine by a pyrimidine or vice versa. Single nucleotide polymorphisms can also arise from a deletion of a nucleotide or an insertion of a nucleotide relative to a reference allele.

[0023] The term "haplotype" refers to the designation of a set of polymorphisms or alleles of polymorphic sites within a gene of an individual.

[0024] A used herein, "plurality" means at least three. In general a plurality of cells, nucleic acid molecules, etc., will contain at least 10, at least about 10^2, at least about 10^3, or at least about 10^4 different cells, molecules, etc.

[0025] A used herein, "entities" refers to a plurality of structurally similar biological molecules or structures (e.g., macromolecules such as nucleic acids, protein, carbohydrates and lipids; cells or subcellular structures or components; viruses) or nonbiological particles that are separate and distinct from each other in the sense that they can be separated into separate reaction chambers using a MPD. "Entity" refers to a single such molecule or structure.

[0026] The term "biological sample", refers to a sample obtained from an organism or from components of an organism, such as cells, biological tissues and fluids. In some methods, the sample is from a human patient. Such samples include sputum, blood, blood cells (e.g., white cells), tissue or fine needle biopsy samples, urine, peritoneal fluid, and fleural fluid, or cells therefrom.

A. Introduction

[0027] The invention relates generally to analysis of macromolecules and small particles, and particularly to analysis of nucleic acids, proteins, and individual cells. In certain aspects, the invention relates to analysis methods involving massive partitioning. Massive partitioning of liquid samples, i.e., dividing the sample into thousands of isolated reaction volumes, has been made possible by the development of specially designed elastomeric microfluidic devices. These devices can be referred to as "massively partitioning devices" or MPDs and are sometimes referred to as "chips" or Digital Isolation and Detection Integrated Fluidic Circuits (DID IFCs). Exemplary devices are described in McBride et al. (PCT publication WO 2004/089,810, published on Oct. 21, 2004; copending, commonly assigned U.S. patent application Ser. No. 10/819,088 published as patent publication No. 20050019792 on Jan. 27, 2005; and copending, commonly assigned U.S. patent application Ser. No. 10/819,088 published as patent publication No. 20050252773 on Nov. 17, 2005, each of which is incorporated by reference in its entirety for all purposes and the specific purposes describe therein and herein; hereinafter referred to together as "McBride et al."). Using MPDs, a sample can be partitioned into a multitude of isolated reaction chambers, and reactions carried out simultaneously in each chamber. For example, McBride et al., supra, describes the performance of 21,000 simultaneous PCR reactions in a single microfluidic chip, in a volume of 90 picoliters per reaction and with single template molecule sensitivity.

[0028] In a first broad aspect, the invention provides new methods and devices for analysis of a sample containing nucleic acids, proteins, other biomolecules, cells, microorganisms, viruses, and other biological or nonbiological entities, in which the sample undergoes massive partitioning as part of the analysis process.

[0029] In a second broad aspect, the invention provides methods and reagents for amplification and/or detection of a nucleic acid. These methods and reagents find particular application in the analysis of nucleic acids partitioned using a MPD, but may be used in amplification-based analysis of any nucleic acid.

[0030] These and other inventions are described in the following sections.

B. Massively Partitioning Devices

[0031] Methods described in this disclosure can be, and in some cases are necessarily, carried out using an elastomeric microfluidic device. Methods for fabricating elastomeric microfluidic devices capable of separating molecules or cells and for carrying out reactions are known in the art (see, e.g., Unger et al., 2000, *Science* 288:113-116, PCT Publications WO 01/01025 and WO/02/43615; and U.S. patent application Ser. No. 10/306,798 published as Pat App. No. 20030138829 on Jul. 24, 2003). In particular, exemplary elastomeric massively partitioning devices (MPDs) are described in McBride et al., supra and references cited therein. Based on these and other publications, one of ordinary skill in the art guided by this disclosure will be able to practice all aspects of the inventions described herein. Accordingly, elastomeric microfluidic devices are described only briefly below.

General Structure of Microfluidic Devices

[0032] Elastomeric microfluidic devices are characterized in part by utilizing various components such as flow channels, control channels, valves, pumps, vias, and/or guard channels from elastomeric materials. FIGS. **1**A and **1**B show an exemplary design of a massively partitioning device.

[0033] A "flow channel" refers generally to a flow path through which a solution can flow. A "blind channel" refers to a flow channel which has an entrance but not a separate exit. A "control channel" is a channel separated from a flow chan-